

Studies on the Biology of Brachy- sporum Trifolii

LEE BONAR

A DISSERTATION
SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE
UNIVERSITY OF MICHIGAN, MAY, 1922

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STUDIES ON THE BIOLOGY OF *BRACHYSPORIUM TRIFOLII*

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In a recent paper (1920) it was shown by the writer that a hitherto undescribed fungus, *Brachysporium trifolii* Kauff., was pathogenic on white clover. During the progress of that study there appeared, in culture, what seemed to be the beginnings of perithecial bodies. This fact led to further studies in order to determine the character and further development of these bodies, and to obtain as complete data as possible regarding the physiological nature of the organism.

The genus *Brachysporium* has not hitherto been the subject of very much laboratory study. On the genus *Helminthosporium*, however, to which *Brachysporium* is very closely allied, a considerable number of papers have appeared, especially along pathological lines, as well as on life-history studies for determining the ascus stage of a number of forms (Hecke, 1898; Ravn, 1901; Diedecke, 1902-1903; Noack, 1905).

The present paper is a record of the studies which were made in an attempt to learn the complete life history of the fungus, and in this way to interpret the significance of the above-mentioned bodies formed in some of the cultures.

This work has been carried on in the laboratories of the Department of Botany at the University of Michigan, under the direction of Prof. C. H. Kauffman to whom are due my whole-hearted thanks for aid and encouragement at all times.

HISTORICAL REVIEW

The intensive study of fungi in culture has long been a subject of interest to mycologists. The method of approach of different workers has varied greatly, both as to point of view and as to methods of study employed.

The first comprehensive discussion of this subject is given by Brefeld (1881) in his "Culturmethode zur Untersuchung der Pilze." In this discussion he points out many of the problems with which one meets in the growing of fungi. He gives a summary of the methods employed in his

own studies, and to some extent of those used by de Bary. These men used, for the most part, either sterilized natural products or extracts or decoctions of these, as substrata for their fungi. They have a very large following among present-day pathological and mycological workers. In many cases satisfactory growth and reproduction have been secured on substrata containing such natural products; but as to the nature and composition of these we can make only rough estimates. Likewise, the growing of fungi in cultures where the food content is definitely known has been practised to a considerable degree. The use of definitely synthesized solutions has been the means of affording us much valuable and definite information.

Pasteur (1858), in his studies on the organisms that cause fermentation, was able to demonstrate the definite rôle played by microörganisms in the chemistry of fermentation. Raulin (1869) published records of the cultivation of fungi on solutions of organic and inorganic salts. Nägeli (1880) contributed a large amount of data on the growth of yeasts and bacteria in synthetic solutions.

The use of some natural products in combination with pure chemical substances of some sort has been very widely employed by a large number of workers during recent years.

The idea of the possibility of finding a universally favorable medium for growth and reproduction in fungi, although often not expressed in a definite statement, seems to have permeated a great deal of the work of recent years. A careful comparison of the physiological reactions of any small number of different organisms will argue very strongly against any such possibility.

During the last decade of the past century there seems to have come a great wave of activity in the study of filamentous fungi in culture. Probably the greatest activity was in the working out of life histories of different forms, especially in the connecting of the ascus and the imperfect stages. Continual use was made of various natural products as food, of synthetic media, and of combinations of these. There came to be a great deal of attention given to the physical factors affecting the development of the organism, such as light, temperature, oxygenation, etc. The chief early exponent and leader in this line of work was Klebs who, in 1896, published his work on algae and fungi, calling attention to the very important rôle played by the external controllable factors in the morphogenesis of the plant and of its organs. The general application of these principles has been frequently demonstrated by workers who have applied them to many different forms and groups of fungi. Beijerinck (1901) showed that the use of food materials of definite composition might be employed as the basis for selective separation of bacteria in mixed cultures. This principle has been very generally used in recent years in bacteriological work. Bachmann (1895) showed the effect of external conditions on spore-formation in *Thamnidium elegans* Link. Klebs (1899, 1913) published comprehensive

summaries of the facts and principles in this field of work, adding new facts and giving an interpretation of the facts that had accumulated. Leininger (1911), working with *Pestalozzia palmarum* Cke., showed that, by the manipulation of the conditions in the culture, one and the same type of spore might be produced either in a pycnidium, in a pseudopycnidium, or on free-growing hyphomycetous conidiophores.

The Saprolegniaceae have been carefully studied by a number of workers; by Klebs (1899), with a single strain of *Saprolegnia mixta*, and by Kauffman (1908, 1921) with different strains of *Saprolegnia mixta* as well as with other species of the family. Kauffman was able to show that the growth and reproduction of these different forms may be controlled by very definite external conditions, and that the potentialities for the development of each form are specific under a given set of conditions. Pieters (1915) brought out the fact that there is no necessary relation between growth and reproduction in the Saprolegnias, and that each process is dependent on a definite set of conditions which are not constant for different forms.

Stevens and Hall (1909) demonstrated that there is a wide range of variation in characters ordinarily used in taxonomic work, when the fungi are subjected to varying conditions of growth in culture. They used a goodly number of forms representing widely divergent groups of plants. They considered the density of the colonies in the culture, the density of the mycelium in relation to zonation on agar media, the chemical composition of the food, and the action of light affecting the cultures.

Coons (1916), by a very intensive study of *Plenodomus fuscomaculans*, showed that in the Sphaeropsidales the physiological reactions of the plants are just as definite and exact as in the groups earlier studied. He gave an especially interesting demonstration of the effect of light on reproduction, and of the nature of this light effect, by showing it to be an oxidation process. Harter, Weimer, and others (1918, 1921) made an application of these principles concerning the effect of varying conditions on the growth of fungi, in their study of the storage rots of the sweet potato. They considered a large number of different sets of conditions to be the optima for the development of different fungi. Their recommendations for preventive measures are based very largely on the idea of an avoidance of these conditions. Leonian (1921), in working out the life history of *Valsa leucostoma*, was able to show the determinate relation of the food material to the production of the ascus-bearing stage of the fungus.

THE FUNGUS, ITS MORPHOLOGY AND CYTOLOGY

A detailed description of *Brachysporium trifolii* Kauff. was given in an earlier paper (Bonar, 1920), but a brief résumé may be given here. This fungus attacks the petioles and leaves of the clover plant, appearing as erect brown conidiophores arising from the mycelium within the tissue of the host. The conidiophores bear from one to several spores at their tips.

The spores are usually 3-septate, with the antepenultimate cell, in most cases, enlarged on one side, thus giving the spore a more or less triangular shape. The spores are dark fuscous brown, and measure 21-31 x 9-11 microns.

A correction in the former description of the fungus should be made at this time. In that publication, a second spore form belonging to the genus *Blennoria* was described as a part of the life history of the *Brachysporium*. It was impossible to isolate this form at that time, and hence it had not been reinoculated into the host to check the evidence for connection. In December, 1921, this *Blennoria* was again found in the greenhouse, this time on plants of the white clover which had not been inoculated with *Brachysporium*. Isolation cultures were made, and the fungus was found to be distinct from *Brachysporium*. Inoculation of clover plants, by spraying with a spore suspension from a pure culture of the fungus, resulted in an infection on about ten percent of the leaves of the plants so treated. Those leaves which were attacked wilted completely, and the typical acervuli appeared on these dying parts which became brown in color. In this manner the fungus, which belongs to the genus *Blennoria* of the *Melanconiaceae*, was shown to be a weak parasite on white clover under greenhouse conditions. This fungus, therefore, must receive an autonomous name, and the combination *Blennoria trifolii* is hereby proposed. For description see the paper referred to (Bonar, 1920, p. 441 and fig. 2, p. 438).

Brachysporium trifolii, when grown on favorable nutrient substrata such as cornmeal agar or on various synthetic agars containing sugars and chemical salts, grows rapidly, and after a few days produces an abundant growth of grayish mycelium. This, as a rule, produces a floccose aërial growth. The typical conidiophores and conidia form in great abundance; frequently, however, the conidiophores are abortive. Conidia are formed in about 6-8 days on the more favorable media. As the conidiophores and spores begin to form, the cultures become darker, in consequence of the darker color of the conidiophores, the spores, and the older massed mycelium.

In cultures on oatmeal agar (formula of Pethybridge and Murphy, 1913) between two and three weeks old, small black bodies appear on the surface of the medium. These grow upward through the mass of aërial mycelium in the form of black columnar stalks. They are a dense black, except the rounded growing tip which is often almost hyaline. The stalks often fork one, two, or three times into an indefinite number of branches. The branching sometimes seems to be dichotomous, while in other cases it is irregular. The bodies may arise singly or in groups, and may be branched or unbranched. They vary in height up to 10 mm., averaging about 6 mm. when developed on oatmeal agar in petri dishes or test tubes. The bodies are at first smooth and are composed of a compact, stroma-like tissue free from any hyphal outgrowths. The older bodies, however, sometimes

become tufted near the apex with brown hyphae. These hyphae, in turn, may bear conidia, or may form simple, hairlike outgrowths (Pl. II, figs. 1a, 1b).

These bodies grow steadily after their appearance in a petri-dish culture, until, after 30-40 days, they present an appearance not unlike a miniature forest set thickly over the surface of the medium. The appearance of these bodies in such abundance on oatmeal agar alone, out of a variety of different media, is a very significant fact. It shows a very marked reaction to the special quality of the food in this substratum. A more careful examination of these bodies shows that they arise from the upper portion of the agar layer, where there has developed an interwoven black mass of hyphae which, however, is not a definite stroma. In section, these younger bodies are seen to consist of pseudoparenchymatous tissue. The outer two or three layers of cells, more especially those of the outer layer, have thick, blackened walls, while the interior tissue is hyaline, with cell walls very like those of the vegetative mycelium in thickness.

The very early stages, when examined in free-hand sections, fail to show any differentiation of tissue in the interior except the differentiation of the black outer wall from the inner hyaline portion. For the further work, paraffin sections were therefore made. A number of different killing fluids and staining methods have been used. The use of Flemming's weak solution and of the triple stain was settled upon as the best treatment for the material in hand. A study of these stained preparations made from the young bodies, 30-35 days old, shows them to be made up of homogeneous tissue. This tissue is composed of very closely interwoven hyphae; in a thin section the cells appear very irregular in shape (figs. 2-4). These cells are also very irregular in size, and those of the first-formed, basal portion are often much larger than those farther up in the body. This tissue arises from a closely interwoven mat of more or less horizontal mycelium in the upper layer of the agar medium, as is shown in figure 2. As emphasized before, this mat is not a true stroma, and, in sections 10 microns in thickness, individual hyphae can be traced for quite a distance through the agar. In other words, they are merely stromatic strands. Preparations made from cultures 45 days old, and from others 60 days old, show, in their interior and a short distance below the tip, a very distinctly differentiated area (fig. 6). The cells within this area are much larger, more loosely connected, and thinner-walled than those of the rest of the body (compare figs. 7 and 8). Furthermore, this area is bounded by a distinct layer of elongated, narrow, very closely packed cells giving the impression of a definite wall around this inner area (Pl. III, fig. 12). In most cases the wall extends completely around this egg-shaped or ellipsoidal area, but in some instances it extends only a part of the way around. In such cases it is dome-shaped above, while at its lower end there is a gradual transition from the enclosed area of the larger differentiated cells to that of the homogeneous

cells which are characteristic of the tissue of the body as a whole. No sharp line or delimiting wall is found, as one moves his point of observation from the inner area downward into the homogeneous body tissue.

If we examine now the cells of the developing columnar bodies in detail, some unusual characteristics appear. The cells of the very young bodies are filled with protoplasm. When this is stained each cell is found to contain from 1 to 5 nuclei, with strands of cytoplasm radiating out from them (Pl. II, figs. 4a, 4b). These nuclei are very small and little can be learned as to their structure. In very clear preparations there is seen within the nucleus a small globular body which is considered a nucleolus. This small particle takes a very brilliant safranin stain, while the remainder of the nucleus is less deeply stained and slightly granular in appearance. As the material becomes older, there is a disappearance of this cell protoplasm until in some cultures 45 days old, and regularly so in 60-day cultures, the compartments stand out as mere walls, void of all cell contents (fig. 7). This disintegration and disappearance of the protoplasm is complete in a great many of the bodies. In some, however, there are found, at this stage, single cells or small clumps of cells which take a very deep stain and hold this stain until all the surrounding tissue is completely destained. These deeply staining cells are found to be considerably larger on the average than those of the surrounding homogeneous tissue. These special cells or clumps of cells may be found singly or scattered through the upper portion of the stalked body. An extensive examination fails to show any connection between the different points of differentiation in a single body where there are such. A great many of these special cells have been examined in an effort to understand the significance of their differentiation. Cases as represented in figures 9a-9c, Plate III, have been found. These show the connection and relation of these larger deeply staining cells with the cells of the homogeneous surrounding tissue. From these figures it is evident that the special cells arise by a differentiation from the ordinary body cells. As before stated, the cells of the body as a whole all contain an abundance of protoplasm when the body is young. Then comes a time in its history when a disintegration of this cellular protoplasm sets in. At special loci, however, the determinant of which we do not know, an exception to this general process occurs. Certain cells are found in or on these intertwined hyphal strands which continue to show an abundance of protoplasm which has a very marked staining reaction. These cells stand out, in thin preparations, in sharp contrast to the surrounding tissue. These differentiated cells by growth and multiplication form clumps of similar cells, as is shown in figures 9a, 9b, and 9c. By continued growth one of these clumps enlarges until we have a stage such as is represented in figure 10. Here can be seen a large number of these cells which are sharply differentiated from the surrounding tissue. Around this specialized area, in section, can be seen a band of narrow, irregularly elongated cells which tend to set this specialized area off from the outer tissue.

These apparently are ordinary body cells which have become flattened and more or less collapsed from the pressure of the growing, expanding group of cells within. The result of this is that a zone of these elongated cells is formed around the inner growing area, and this zone forms the delimiting wall such as is shown in figure 12 in an older case. The growth and expansion of this inner area continues until a certain degree of development is reached or a certain set of conditions prevail, beyond, or on account of, which, further differentiation does not occur. Instead of further growth, a process of disintegration then sets in within the protoplasm of the cells in this area. In figure 11 are seen some cells which contain their normal nuclei and cytoplasm, while in others there has been a disintegration so that the cell is empty, in part. In still other cells it will be seen that there has been a complete disappearance of the protoplasm, and the cell wall alone remains. In older material large numbers of the differentiated areas are found, within which the cells are completely lacking in any protoplasmic content (fig. 12). These empty cells of the differentiated area are very different, however, from those which make up the surrounding general tissue of the stalked body as a whole. This is best brought out by a comparison of figure 7 with figure 8 (Pl. II). These figures are both taken from the section shown as a whole in figure 12. Figure 7 is an enlarged view of a portion of the tissue outside the central differentiated area. Figure 8 is an equal magnification of a portion of the inner area and is separated from that of figure 7 by the wall, only a few cells in thickness.

The time, in the life history of a culture, of the beginning of these differentiated areas in the stalked bodies is rather uncertain. Material from 30-day cultures does not show any differentiation, and most of the cells of the stalked body show stainable protoplasmic contents. Material from 45-day cultures shows specialized deeply staining cells or small clumps of these cells. Figure 10 was made from material from a 4-months-old culture, but other preparations from 4-months-old cultures have been studied which show the degree of development represented in figure 12. This process is apparently very much affected by the conditions under which the cultures are developed, as are the other life processes of this organism.

CULTURE METHODS AND MEDIA

Brachysporium trifolii has been grown on a large number of different kinds of media. Methods for their preparation and the formulae are given below. What are spoken of as agars were used in test tubes and petri dishes. For solution cultures, 25-cc. loose-cover glass capsules each containing a cone of filter paper, such as were described by Coons (1916), were used at first. The most favorable cultures were secured, however, by the use of filter-paper cups in capsules, as described by Leonian (1921). Into these filter-paper cups—made by pushing in the moistened tip of a filter-paper cone with the finger—any kind of food materials could easily be placed; as,

for example, agars, corn meal, wheat flour, gluten, rice, or rice paste, which were used in this way. These cultures were prepared and then sterilized in the autoclave. The water supply was kept constant by keeping sterile water in the bottom of the capsule. The filter paper served as a wick to supply a constant moisture to the material in the cups. Genuine Whatman no. 1 filter paper, made by W. and R. Ralston, was used for all these cultures. Distilled water was used for all preparations.

The media were prepared as follows:

Melilotus stems: green stems of *Melilotus* cut in sections 3 inches long. Put in test tubes with a little water, and autoclaved at 15 lbs. for 20 min.

Green beans: green string-bean pod put in test tube with a little water, autoclaved at 15 lbs. for 20 min.

Potato plugs: cylinders of fresh potato in test tube with a little water, autoclaved at 15 lbs., 20 min.

Carrot plugs: same as potato plugs.

Rice grains, rice paste, wheat flour, gluten, pure wheat starch, and corn meal: put material in filter-paper cups in capsules with a little water in bottom of capsule, autoclaved at 15 lbs., 20 min.

Cornmeal agar: 4 teaspoonfuls cornmeal, 1000 cc. water, cook in double boiler at 60° C. for 1 hour. Strain through gauze and add to filtrate 15 g. agar: heat until agar is dissolved. Filter through filter paper, autoclave at 15 lbs., 20 min.

Prune-juice agar: cook 200 g. prunes in 500 cc. water slowly for 1 hr. Restore to original volume, add 500 cc. water in which is dissolved 15 g. agar. Clear with white of egg, filter, autoclave at 15 lbs., 30 min.

Duggar's agar (Duggar, "Fungous Diseases of Plants," p. 26): NH_4NO_3 1.0 g., MgSO_4 0.25 g., KH_2PO_4 0.5 g., FeCl_3 trace, cane sugar 5.0 g., water 100 cc., agar 1.5%. Dissolve, clear, and filter through filter paper. Autoclave at 15 lbs., 15 min.

Duggar's agar and peptone: same as above, plus 0.2% peptone.

Oatmeal agar or "oat agar": grind 60 g. Quaker oats to fine powder. Stir into 1000 cc. cold water. Cook in double boiler with frequent stirrings 10 or 15 minutes. Add 10 g. agar. Continue heating until agar is dissolved. Stir constantly. Pour into large test tubes and autoclave at 15 lbs., 45 min.

Light oat agar: same as above except that 30 g. oatmeal is used instead of 60 g.

Water extract of oatmeal: 60 g. oatmeal in 1000 cc. water. Let stand in cool place for 24 hrs. with occasional stirring. Strain through cloth, filter through paper, autoclave at 15 lbs., 20 min.

Leonian's agar: water 1000 cc., KH_2PO_4 1.25 g., MgSO_4 0.625 g., maltose 6.25 g., malt extract 6.25 g., agar 15 g. Dissolve salts in 500 cc. of water. Dissolve agar in other 500 cc., mix the two, clear, filter, autoclave at 10 lbs. for 15 min.

Leonian's solution: use solution as above without agar.

Coons' solution: use as materials M/5 solutions. For 100 cc. nutrient solution, take MgSO_4 1 cc., KH_2PO_4 5 cc., asparagin 5 cc., maltose 5 cc., water 84 cc., autoclave at 10 lbs., 15 min.

Modification of Coons' solution: same as above, except that M/5 levulose is substituted for M/5 maltose.

Agars plus carbohydrates: to the prepared and melted agar is added, and intimately mixed, before sterilization, the desired percentage of the carbohydrate.

Solution e:

Dihydrogen potassium phosphate	M/20 sol., 50 cc.
Calcium nitrate	M/20 sol., 50 cc.
Levulose	4 percent, 50 cc.
Magnesium sulphate	M/20, 2 cc.

Solution *f*: Solution *e*, using 10 percent levulose instead of 4 percent.

Solution *g*: Solution *e*, plus 50 cc. of 4 percent malt extract.

Solution *h*: Solution *g*, plus 50 cc. 0.2 percent peptone.

Solutions, *e*, *f*, *g*, and *h*, as well as various sugar solutions, were autoclaved at 10 lbs. for 15 min.

In the sterilization of media I followed the idea put forward by Mudge (1917), that a high temperature for a short time has less effect in hydrolyzing proteins and sugars than does a lower temperature for a longer time.

EXPERIMENTAL STUDIES

The bodies which have been previously described under "Morphology and cytology" appeared early in the study of the fungus. The first cases of differentiation observed were of a stage such as represented in figure 12, Plate III. The question of their significance in the life history of the organism was not easily answered. The most logical conclusion to be drawn at that time was that these differentiated areas represented an incomplete development of some reproductive body, possibly a perithecium. As a starting-point, a number of experiments were set up to analyze the following factors: first, conditions which might promote further development in these differentiated areas; second, the presence of possible inhibitory influences, which might arrest a further differentiation in these areas; and third, specific physiological reactions of the plant toward controlled environmental conditions. Data obtained from these experiments have been arranged according to: (1) vegetative or mycelial growth; (2) conidium-production; (3) the production of the stalked bodies. These data are given where possible in tabular form by the use of symbols to represent degrees of development. The Roman numerals, O, I, II, III, IV, and V are employed to represent varying degrees of development. O, for example, means no development, and V the maximum development of the process under consideration.

Tables 1, 2, and 3 classify these substrata with regard to the degree of development which they induce in each of the above-named processes of the plant. The factors influencing these different processes are discussed separately following each of the three tables.

FACTORS INFLUENCING MYCELIAL GROWTH

Physical Factors

Temperature

One of the most important factors in the growth of any plant is temperature. Mention is made of the effect of temperature on the growth of this fungus in the earlier publication (Bonar, 1920). Later checking up of these results and records of further study are given here. The lower limit for growth is between 6° and 8° C. Growth is slow and long-continued between

8 and 16 degrees. Growth is much more extensive and rapid at a higher temperature up to its optimum, which is between 20° and 24° C. As the temperature is raised above 24° C. (providing humidity is favorable), the growth gradually becomes less extensive, until at 30° C. 1-month-old cultures show a small growth colony 1 cm. in diameter on the more favorable media.

TABLE I. *Degree of Vegetative Growth in Different Substrata*

O	I	II	III	IV	V
	Sol. <i>e</i> and M/1 NaCl	Gluten	Pot. plugs	Melilotus stems	Pure wheat starch
	Sol. <i>e</i> and 2M NaCl	Beef-infusion agar	Green beans	Whole rice	Cornmeal
	Oat-agar cup and 2.5M NaCl	Duggar's agar and 0.2% peptone	Carrot plugs	Wheat flour	Cornmeal agar
	Oat-agar cup and 3M NaCl	Water extract of oatmeal	Rice-flour paste	Pot. agar	Prune-juice agar
		Maltose 1%	Duggar's agar	Light oat agar	Leonian's agar
		Glucose 1%	Oat agar, 10% agar	Pot. agar and 1% levulose	Oat agar
		Oat agar and 1.5 M NaCl and 1% lev.	Glucose 5%	Pot. agar and 1% sucrose	Oat agar and 1% sucrose
		Oat agar and 2M NaCl and 1% lev.	Levulose 1%	Pot. agar and 1% glycerin	Oat agar and 1% levulose
	Sol. <i>e</i> and M/2 NaCl		Sol. <i>f</i>	Oat agar and 1% glycerin	Oat agar and 1% glucose
			Sol. <i>h</i>	Oat agar and 1% lev. and 1% glycerin	Oat agar and 1% maltose
			Coon's sol.	Sol. <i>e</i>	Cornmeal agar and 1% levulose
			Plain agar cups and Coons' sol.	Sol. <i>g</i>	Light oat agar and 1% lev.
			Oat agar and M/1 NaCl and 1% lev.	Mod. of Coons' sol.	Leonian's sol.
			Sol. <i>e</i> and M/5 NaCl	Oat agar and KH ₂ PO ₄ and 1% maltose	Oat agar and KH ₂ PO ₄ and 1% lev.
				Oat agar and Coons' sol.	Oat agar and sol. <i>e</i>
				Oat agar and mod. Coons' sol.	Oat agar and KH ₂ PO ₄
				Plain agar cups and sol. <i>e</i>	Oat agar and NaH ₂ PO ₄
				Plain agar cups and mod. of Coons' sol.	Oat agar and sol. <i>e</i> -MgSO ₄
				Oat agar and M/5 NaCl and 1% lev.	Oat agar and M/2 NaCl and 1% lev.
				Oat agar and M/2 NaCl and 1% lev.	Oat agar and M/10 NaCl and 1% lev.
				Sol. <i>e</i> and M/10 NaCl	

The effect of temperature on the time of spore germination is very marked, as is shown in the following experiment. Spores were placed in hanging-drop cultures in water, in Van Tieghem cells, and these were subjected to different temperatures. The length of time necessary for the formation of a well differentiated germ tube was noted.

Spores at 0° C.	No germination after 8 days.
" " 10° C.	Germinated in 18-36 hours
" " 22° C.	" " 4-9 "
" " 25° C.	" " 3-5 "
" " 30° C.	" " 2-3 "

The time given represents the range from the time when any single spore was seen to be germinating until more than 50 percent of the spores in the suspension had been germinated. Spores kept 8 days at 0° C. germinated in 1 to 2 hours when transferred from 0° C. to 30° C.

Light

In the study of this point, different types of cultures were grown in diffuse light and in the dark for varying lengths of time, but no marked difference could ever be detected between them. Cultures grown by a window, where they were subjected to the direct rays of the sun for a few hours each day, produced less extensive growth of mycelium than those in diffuse light or in the dark.

Oxygen Requirements

The mycelium of this fungus requires an abundant supply of oxygen for growth. This is shown by the following experiment. Test-tube cultures when three days old, showing a tuft of vigorously growing mycelium, were sealed by dipping the plugs into melted paraffin. Cultures of the same series were kept unsealed as checks. The growth continued in the sealed tubes for 1 to 2 weeks, and in that time did not cover the agar slant in the tube. After two weeks no perceptible growth occurred in the sealed tubes, while the cultures in the unsealed tubes continued to grow and covered the whole of the surface of the slant with a heavy mat of mycelium.

Humidity

The degree of humidity in the culture chamber has a very evident effect on growth. In the preliminary report it was stated that no growth was secured at 26 degrees C. The medium then used was oatmeal agar in petri dishes, placed in an incubator. Further studies on this point reveal the fact that in a dry atmosphere growth ceases at from 26 to 28 degrees C., while in an atmosphere approaching saturation, and on favorable substrata, growth may occur at higher temperatures. Cultures on filter-paper cones, set with their bases in a nutrient solution in loose-cover capsules, show a slight growth at 30 degrees C. These colonies are from 1 to 2 cm. in diameter at the end of one month, and the mycelium is composed, in large part, of irregular clumps of heavy-walled, dark, rounded cells.

Physico-chemical Factors

There are certain classes of chemicals, the presence or the absence of which, although they do not act as foods, may affect the growth of the fungus in culture in a very decided manner. Klebs (1899) makes the generalization that fungi as a rule grow best on slightly acid media. Coons (1916) says: "It is commonly said that fungi grow best under slightly alkaline conditions." It matters little whether we try to make generalizations or not, we must always consider the specific requirements of the organism under consideration, and these will frequently be found to be different from the requirements of many other organisms. The plant here studied shows a rather wide tolerance to either acidity or alkalinity in relation to its vegetative growth. A number of titrations made on oatmeal agar, which is the optimum substratum for this fungus, show an average reaction¹ of + 1. Titrations on a number of the other media, as they are made up, show them as a rule to be slightly acid in their reaction.

To test the reaction of the plant under study, five sets of cultures on oatmeal agar were made up, in which the reaction was adjusted as follows: - 1, neutral, + 1, + 2, and + 3. (In the last-cited the agar failed to jell because of the excess of acid.) Growth in -1 was in the form of a thin, closely packed felt over the surface, and was of slight amount; in neutral there was very much more growth with a fluffy aërial mycelium; in + 1, which proved to be the optimum, a very vigorous and extensive growth occurred: in + 2 the growth was very like that in neutral, while in + 3 it was very much reduced but slightly more extensive than in -1. Thus it is shown that this fungus, while not very sensitive to slight changes in acidity, grows best on a slightly acid substratum.

Quality of Food

This fungus grows, as is shown in table 1, on a very large number of different kinds of substrata. It is seen from this table also that a favorable degree of vegetative growth occurs on a large number of these. Most important for vegetative growth, it seems, is the presence of a plentiful supply of carbohydrate material, as will be seen from a study of column V of the table. There are, however, specific relations between the extent of growth and the nature of the different carbohydrates used as food. Pure starch from wheat gives a very abundant growth. 1 percent solutions of glucose or maltose do not produce so extensive a growth as does a solution of levulose of like concentration. Those natural products like cornmeal, oatmeal, and rice, or products of these, contain, besides an abundance of carbohydrates, a supply of proteins and certain salts, and all produce a vigorous

¹ The acidity or the alkalinity of the medium is expressed in terms of that quantity, in cc., of a normal (N/1) volumetric solution which would bring 100 cc. of it to the neutral point, using phenolphthalein as an indicator. If acid, the plus sign (+) is placed before the number expressing this amount; if alkaline, the minus sign (-) is used instead.

growth when used as media. Those substances containing a high percentage of nitrogen with a low carbohydrate content are unfavorable for vegetative growth. Examples of these are beef-infusion agar, which contains an abundance of animal protein with very little carbohydrate material, and gluten, which contains only the proteins from wheat flour. The specific nature of the nitrogenous matter is also effective in influencing growth. Leonian's solution, having as its nitrogen supply malt extract, is much more favorable for growth than Coons' solution, in which asparagin is used. An appreciable reduction in growth occurs when to Duggar's agar is added 0.2 percent peptone. These different media all contain the salts which are necessary for growth, but the chief factors influencing the amount of growth seem to be combinations of certain carbohydrates and proteins.

Quantity of Food

The quantity of food is a less important factor than is the quality of food, in the production of mycelial growth in this fungus. All the media used are comparatively rich and far above the concentration minimum for growth. In a study of table I, however, there appear some instances in which the quantity of the food shows an appreciable effect. A 1 percent solution of glucose gives a growth of II, while a 5 percent solution gives a growth of III. Water extract of oatmeal gives a growth of II, while oatmeal gives a growth of V. The osmotic concentration of the nutrient solution is also an important factor. Solution *f* gives a growth of III as compared to a growth of IV for solution *e*. The two solutions are identical except in the percentage of levulose present. In solution *e*, 4 percent levulose is used. In solution *f*, 10 percent levulose is used. Other examples of the effect of concentration are seen in those cultures to which varying amounts of NaCl have been added. Oatmeal-agar cups to which had been added 1 percent levulose solution were used as checks in these experiments. Varying amounts of NaCl were added to this levulose solution. When NaCl is added to the levulose solution in quantities to make an M/20 or M/10 solution of NaCl, the amount of growth is not affected. When more than these amounts are added there is effected a reduction in growth until in 2 M or 3 M solution the growth is represented by I. In such extreme cases the growth of the fungus is almost entirely composed of heavy-walled, rounded chlamydospores in irregular clumps or masses. Growth in nutrient solution *e* is more seriously affected by the addition of NaCl than are those cultures on agar. Cultures on solution *e* show a reduction of growth in concentrations of M/1 to 2 M NaCl equal in degree to that shown in 2 M to 3 M solutions which have been added to oatmeal agar in cups. The nature of the growth in these solutions varies from a much-branched clear mycelium in solution *e*, or solution *e* plus NaCl to make an M/10 solution, to irregular clumps of thick-walled, black chlamydospores in M/1 or 2 M solutions of NaCl in solution *e*.

TABLE 2. *Degree of Conidium-production on Different Substrata*

O	I	II	III	IV	V
Potato plugs	Pot. agar and 1% glycerin	Green beans	Carrot plugs	Wheat flour	Melilotus stems
1% maltose	Oat agar and 1% lev. and 2 M NaCl	Gluten	Whole rice	Pure wheat starch	Cornmeal
1% glucose	Oat agar and 1% lev. and 2 M NaCl	Sol. <i>f</i>	Rice: flour paste	Sol. <i>e</i>	Cornmeal agar
1% levulose	Sol. <i>e</i> and M/2 NaCl	Leonian's agar	Sol. <i>g</i>	Prune-juice agar	Oatmeal agar
5% glucose		Duggar's agar and 0.2% peptone	Sol. <i>h</i>	Light oat agar	Oat agar and 1% lev.
Beef-infusion agar		Water extract of oatmeal	Coons' sol.	Oat agar and 1% glucose	Oat agar and 1% sucrose
Oat agar and 1% lev. and 2.5 M NaCl			Mod. of Coons' sol.	Light oat agar and 1% lev.	Cornmeal agar and 1% lev.
Oat agar and 1% lev. and 3M. NaCl		Pot. agar and 1% lev.	Pot. agar	Oat agar, 1% maltose, and KH_2PO_4	Oat agar and 1% lev.
Sol. <i>e</i> and 1.5 M NaCl		Pot. agar and 1% sucrose	Duggar's agar	Oat agar, Coons' sol.	and M/20 KH_2PO_4
Sol. <i>e</i> and 2 M NaCl		Oat agar and 1% lev. and M/1 NaCl	10% oatmeal agar	Oat agar and sol. <i>e</i> — MgSO_4	Oat agar and mod. of Coons' sol.
		Sol. <i>e</i> and NaCl	Oat agar and 1% maltose	Plain agar cups and Coons' sol.	Oat agar and sol. <i>e</i>
			Oat agar and 1% glycerin	Plain agar cups and mod of Coons' sol.	Oat agar and M/20 KH_2PO_4
			Oat agar and 1% lev. and 1% glycerin	Oat agar and 1% lev. and M/5 NaCl	Plain agar cups and sol. <i>e</i>
			Oat agar and 1% lev. and M/2 NaCl	Sol. <i>e</i> and M/10 NaCl	Oat agar and M/20 NaH_2PO_4
					Oat agar and 1% lev. and M/20 NaCl
					Oat agar and 1% lev. and M/10 NaCl

FACTORS INFLUENCING CONIDIUM-PRODUCTION

Table 2 classifies the various substrata with reference to the degree of conidium-formation they induce. The estimates of the degree of conidium-formation are based on the average number of spores found in a number of fields under the microscope, using as nearly as possible uniform bulk of material and uniform dilution in preparing the amount.

Physical Factors

Temperature

The optimum temperature for conidium-production is the same as that for growth, 20°–24° C. The minimum temperature for conidium-production is 15°–16° C. The maximum is, under other optimum culture conditions, 27° C. The cultures used for the compilation of the tables were grown at the optimum temperature of 22°–25° C.

Light

No perceptible effect on the production of conidia is produced by keeping in either the light or the dark.

Oxygen Requirements

Conidium-production is reduced, by the exclusion of oxygen, to even a greater degree than is mycelial growth.

Humidity

The humidity of the culture chamber influences the amount of conidium-production to a fairly marked degree. In cultures where the atmosphere is very dry, production is inhibited. In cultures which afford a moist substratum, such as Melilotus stems, there is a very abundant conidium-production with a comparatively low degree of moisture in the culture chamber. In culture chambers which approach saturation, fewer conidia are produced; here the conidiophores are not formed in a regular manner, but a fluffy aërial mycelium develops with abortive conidiophores.

Physico-chemical Factors

Acidity or alkalinity of the culture medium is also a factor in the production of conidia.

Cultures with reaction of -1 are marked O for conidium-production.

Cultures with reaction of 0 are marked III for conidium-production.

Cultures with reaction of $+1$ are marked V for conidium-production.

Cultures with reaction of $+2$ are marked III for conidium-production.

Cultures with reaction of $+3$ are marked I for conidium-production.

Quality of Food

A study of table 2 at once shows that the quality of the food is a very important factor in the production of conidia. In solutions of carbohydrates no conidia are produced. Substrata which are very rich in proteins, as green bean pods, gluten, Duggar's agar and peptone, fall in column II for conidium-production. Those media which afford more of a balance of carbohydrate material and nitrogenous material, either organic or inor-

ganic, are the more favorable for conidium-production, as is seen by referring to columns IV and V. Materials having a low protein content, as polished rice and potato agar, show a low production of conidia, and the addition of a sugar solution, as levulose, does not stimulate conidium-production. A marked difference in conidium-production is shown when different sugars are added to a culture, either in an agar or as a solution to a filter-paper cone or cup. In oatmeal agar plus levulose a maximum number of conidia are produced, while oatmeal agar plus maltose in like percentage rates III in conidium-production. The addition of 1 percent of glycerin serves to reduce conidium-production on oatmeal agar from V to III and on potato agar from III to I.

Quantity of Food

The production of conidia is affected by the quantity of available food also. On oatmeal agar conidium-production is rated as V; on light oatmeal agar, which contains half as much oatmeal, conidium-production is rated IV; on water extract of oatmeal, conidium-production is rated II. Conidium-production on solution *e* falls in column IV, while that on solution *f* falls in column II. Solution *f* is different from solution *e* in that it calls for 10 percent levulose where *e* calls for 4 percent levulose. An interesting series of results are obtained from those cultures to which were added varying amounts of NaCl. Conidium-production is reduced on oat-agar cup cultures when the levulose solution in the capsule contains more than M/10 concentration of NaCl, and completely inhibited by more than 2 M concentration. Cultures on solution *e*, to which NaCl was added, are more sensitive. No reduction in conidium-production is produced by M/10 concentration of NaCl. M/1 concentration completely inhibits conidium-formation. Thus the filter-paper cone cultures are seen to be more sensitive to the concentration or toxic effect than are the oatmeal-agar cup cultures.

These results are at variance with those of Klebs on *Eurotium repens* (1896), wherein he demonstrated that conidium-production was most abundant on 15 percent solution of glucose. A reduction of this concentration caused a reduction in conidium-production. However, when he added NaCl to the solution in quantities sufficient to maintain an osmotic pressure equal to that of the 15 percent glucose solution, the maximum of conidium-production was maintained with a very small amount of glucose. In my cultures there is an evident toxicity with NaCl in concentrations above M/10. As this concentration is increased the toxic effect is more pronounced, until complete inhibition of conidium-formation is obtained. A reduction below this toxic minimum of an M/10 concentration produces no reduction of conidium-formation, which proves that the effect is not a concentration effect, but a specific toxicity.

The effect on conidium-production of a sudden removal of all or most of the food supply is demonstrated by the following experiment. Six filter-

paper cone cultures containing solution *g*, and six containing solution *h* were inoculated and allowed to grow for one month. At that time an examination was made to determine the degree of conidium-production. Under the low power of the microscope in specimens from cultures in solution *g*, the average number of conidia per field was thirty. In similar ones from solution *h*, the number averaged about twenty-five per field. Each of the filter-paper cones was then transferred by sterile forceps to large 200-cc. capsules containing sterile distilled water, and allowed to stand for thirty minutes. At the end of that time, of those from solution *g*, three were put in small capsules containing a fresh supply of solution *g*. The other three were put in small capsules containing sterile distilled water. The six cultures from solution *h* were handled in a like manner, three being put into fresh solution *h* and three into distilled water. These were again examined one month after these transfers were made. Those cultures which, after the washing in distilled water, were returned to fresh quantities of the nutrient solutions, were identical with the checks which had remained undisturbed in the nutrient solutions. There was no apparent increase in the amount of conidium-production during the second month. Those cultures which had been transferred to distilled water showed a very marked increase in conidium-production. Mounts from cultures remaining in solution *g* gave an average of 30 conidia per field of the microscope. Those from cultures which had been transferred from solution *g* to distilled water showed 100-150 per field in similar mounts. Mounts from cultures remaining in solution *h* showed an average of 25 conidia per field. Those which were transferred from *h* to distilled water showed from 100 to 200 conidia per field in similar mounts. It is seen from these results that those cultures which were grown in solution *h* produce, when transferred to distilled water, a greater percentage increase in conidium-production than do those from solution *g*.

The outstanding fact is that the transferring of culture growths from a rich solution to distilled water stimulates conidium-production to a very marked degree. This fact was first well demonstrated by the work of Klebs on *Saprolegnia mixta* in 1899. He showed that the nature of any specific development is conditioned by the environment to which the plant is subjected. Mention is made earlier in this paper of a number of other workers who have further demonstrated the validity of this principle. Pieters (1915) maintained that "the effect of a given environment may not become evident until the plant has been transferred to another environment." This is supported by the above-described experiment. Solution *h* equals solution *g* + 0.2 percent peptone. Vegetative growth in solution *g* is graded IV, while that in solution *h* is graded III. (See table 1). Although vegetative growth is less extensive in solution *h* than in solution *g*, the inverse is true of conidium-production when cultures in the two solutions are transferred to distilled water. The addition of peptone therefore

seems to be a stimulating factor which finds expression in an increased conidium-production after the culture has been transferred to distilled water, while it does not find such expression in those cultures which are kept in the rich solution.

TABLE 3. *Degree of Production of Stalked Bodies in Different Media*

O	I	II	III	IV	V
Pot. plugs	Green beans	Melilotus stems	Whole rice	Oat-agar cup and sol. <i>e</i> —MgSO ₄	Oat agar
Carrot plugs	Cornmeal	Rice paste	Wheat flour	Oat-agar cup and sol. <i>e</i> (Na for K)	Oat agar and 1% lev.
Pure wheat starch	Sol. <i>f</i>	Gluten	Sol. <i>e</i>	Plain agar cups and sol. <i>e</i>	Oat agar and 1% sucrose
Maltose 1%	Sol. <i>g</i> Coons' sol.	Pot. agar	10% oat agar		Oat-agar cup and M/20 KH ₂ PO ₄
Glucose 1%	Mod. of Coons' sol.	Oat agar and 1% glucose	Oat-agar cup and mod. Coons' sol.		Oat-agar cup and 1% lev. and M/20 KH ₂ PO ₄
Levulose 1%	Prune-juice agar	Oat agar and 1% maltose	Oat-agar cup and 1% lev. and M/10 NaCl		Oat-agar cup and sol. <i>e</i>
Glucose 5%	Light oat agar	Pot. agar and 1% lev.			Oat-agar cup and 1% lev. and M/20 NaCl
Sol. <i>h</i>	Cornmeal agar and 1% lev.	Pot. agar and 1% sucrose Oat-agar cup and 1% maltose and M/20 KH ₂ PO ₄			
Leonian's sol.	Light oat agar and 1% lev.				
Water extract of oatmeal	Plain agar cups and mod. of Coons' sol.	Oat-agar cup and Coons' sol.			
Beef-infusion agar	Sol. <i>e</i> and M/10 NaCl	Oat-agar cup and 1% lev. and M/5 NaCl			
Cornmeal agar					
Duggar's agar					
Duggar's agar and 0.2% peptone					
Leonian's agar					
Pot. agar and 1% glycerin					
Oat agar and 1% glycerin					

TABLE 3. *Continued.*

O	I	II	III	IV	V
Oat agar and 1% levulose and 1% glycerin					
Plain agar cup and Coons' sol.					
Oatmeal-agar cup and 1% lev. and M/2 NaCl					
Oat-agar cup and 1% lev. and M/1 NaCl					
Oat-agar cup and 1% lev. and 1.5 M NaCl					
Do. and 2 M NaCl					
Do. and 2.5 M NaCl					
Do. and 3 M NaCl					
Sol. <i>e</i> and M/5 NaCl					
Sol. <i>e</i> and M/2 NaCl					
Sol. <i>e</i> and M/1 NaCl					
Sol. <i>e</i> and 1.5 M NaCl					
Sol. <i>e</i> and 2 M NaCl					

FACTORS INFLUENCING THE PRODUCTION OF STALKED BODIES

Temperature

The limits of temperature within which the growth of the stalked bodies occurs are rather narrow. None appear in cultures held consistently below 20° C. or in cultures maintained at temperatures above 25° C. The optimum temperature ranges from 22°–24° C. Cultures kept constantly at 25° C. have not as large and vigorously growing bodies as those at 22°–24° C. As one approaches either the temperature minimum or temperature maximum for these bodies, they become fewer in number and smaller.

Light

Light, here as in the other phases of the life history of the plant, seems to be of rather small importance as an influencing factor. Cultures grown in the dark, however, show a greater number of vigorously growing bodies than do those grown in the light.

Oxygen Requirements

Cultures from which a free circulation of air is excluded by sealing with paraffin fail to show any sign of the production of these stalked bodies.

Humidity

Humidity of the culture chamber is not so much a factor in the number of the bodies developed as in the degree of development which they attain. On oatmeal-agar cultures in test tubes, which become comparatively dry after 50 to 60 days, the bodies seldom attain a height of more than 6 mm. In filter-paper cups containing this same agar, and a supply of moisture in the bottom of the capsule, the development of the bodies is more continued and extensive. In some of these cultures bodies are found which measure 10 mm. in height. This greater size is due, in part at least, to the maintenance of a plentiful supply of moisture.

Physico-chemical Factors

The acidity or alkalinity of the medium very effectively limits the growth of these bodies. The reaction limits within which they may grow are wide enough, however, to include most common laboratory media.

In media with a reaction — 1, no bodies are produced.

In media with a reaction neutral, very few bodies are produced.

Media with a reaction + 1 are optimum.

In media with a reaction + 2, very few bodies are produced.

In media with a reaction + 3, no bodies are produced.

Quality of Food

By far the most important factor in the production of these stalked bodies is the quality of the food materials. This is best brought out by a consideration of table 3. When compared with tables 1 and 2, it is seen that the number of different media which produce an optimum of growth of these bodies is very small in comparison with the number which are optimum for the other functions of the plant under consideration.

The outstanding fact to be observed in table 3 is that those media listed in column V all contain one essential substance, *viz.*, oatmeal agar. This oatmeal contains a specific combination of materials most favorable for the development of these bodies. The addition of other substances, such as levulose and salts, in some cases promotes and prolongs the vigor of growth to a small degree, but the effect is that of an increased or stimulated vigor of the plant as a whole rather than a specific effect on the production of these bodies. This was brought out by a series of cultures which were set up, using an M/20 solution of various salts, both with and without the presence of sugars. These salt solutions were added to oat-agar cup cul-

tures and grown for 3 to 6 months. The salts used were NaCl , K_2SO_4 , KH_2PO_4 , KNO_3 , $\text{Ca}(\text{NO}_3)_2$, NaH_2PO_4 , and $\text{Ca}_3(\text{PO}_4)_2$. These were added to cultures containing 1 percent levulose solution in one series and 1 percent sucrose in a second series. The bodies were produced in all the cultures. No difference could be seen in the growth on cultures to which was added 1 percent levulose alone, $\text{M}/20$ KH_2PO_4 alone, or a combination of the two. These did, however, show a more extensive development than those cultures on oatmeal alone. Of the other six salts used, none resulted in the production of quite so vigorous bodies as KH_2PO_4 . K_2SO_4 and $\text{Ca}_3(\text{PO}_4)_2$ appeared least favorable for the production of the bodies, and afforded a less abundant production of the bodies than oatmeal agar alone.

The addition of 1 percent glycerin to the most favorable media, *viz.*, oatmeal-agar cups and 1 percent levulose, completely inhibits the growth of the bodies.

Levulose is the most favorable sugar for the stimulation of growth of the bodies. Sucrose is very nearly as good as levulose, but, considering the average over a very large number of cultures, it is found to be slightly less effective in inducing development of the bodies. Maltose is decidedly less favorable than either levulose or sucrose. Glucose is on a par with maltose and they both fall in column II, table 3, while levulose and sucrose fall in column V. This correlation between maltose and glucose, and sucrose and levulose, is quite to be expected, since maltose hydrolyzes into 2 molecules of glucose, and sucrose into 1 molecule of levulose and 1 of glucose. That this effect of the addition of levulose is purely a developmental stimulus, and not a formative one, is brought out by adding levulose to other media besides oatmeal agar, and by using it in solutions by itself. No bodies are produced on levulose solution, nor on any other pure sugar solutions. On cornmeal agar and levulose an occasional small body is produced. On potato agar a few bodies appear. The addition of levulose to the medium does not increase the number of bodies produced.

The nitrogenous content of the substratum used seems to be the most important factor in the production of the bodies. There is a specific protein content in oatmeal which makes it the most favorable of any substance used for the production of the bodies. Wheat flour (ordinary process), potatoes, and cornmeal are all rich in carbohydrates, but less rich in proteins than is oatmeal. On wheat flour, bodies are produced in smaller numbers, and these attain a lower degree of development, than do those on oatmeal. That the protein is the formative influence in the production of these bodies is demonstrated by the following experiment: The gluten was kneaded out of some flour from the same sample used for culture cups. This gluten was made as free from starch as possible. It was sterilized in filter-paper cups. At the same time, some pure wheat starch was placed in filter-paper cups and sterilized. These preparations were inoculated with the fungus. On the gluten cultures there was a sparse mycelial growth and

numerous stalked bodies were started. These did not develop well, however. In no instance did they attain a height of more than 1 mm. On the starch cultures there was an extensive mycelial growth, but no sign of the bodies. The replacing of the water in the gluten-cup cultures by 1 percent levulose solution did not induce any appreciable increase in the development in the bodies, although it did increase vegetative growth. Thus it appears that for the development of these bodies there must be present, along with a specific nitrogenous food content, some starch-bearing substance.

On synthetic solutions there is a marked variation in the production of these bodies. They are in no instance as favorable as is the oatmeal agar. Solution *e*, which falls in class III, is most favorable for the production of bodies of any solution used. Coons' solution falls in class I. Modification of Coons' solution falls in class I. Solution *e* employs as a nitrate $\text{Ca}(\text{NO}_3)_2$, while the modification of Coons' solution employs asparagin; otherwise the two solutions contain the same materials. Leonian's solution employs maltose and malt extract, both of which tend to inhibit body-formation, and in it no bodies at all are produced. The addition of malt extract to solution *e* reduces its rating for the production of bodies from III to I.

Quantity of Food

The influence of the quantity of food on the production of these bodies is best brought out by a comparison of the development on the standard oatmeal agar, and on that which contains one half the amount of oatmeal in the standard. In the standard, 60 grams of oatmeal are added to one liter. In the so-called light oatmeal agar, 30 grams of oatmeal are added to a liter. The production of bodies on the standard is V, while that on the light agar is I. The concentration of other added elements to the oatmeal agar may cause variations. The making of a 10 percent agar instead of the regular 1 percent concentration reduces body-production from V to III on an otherwise identical medium. The use of 10 percent levulose solution in preparing solution *f*, as compared with a 4 percent solution used in preparing solution *e*, reduces body-formation from III to I.

The production of these bodies is inhibited by a lower concentration of toxic substances than that affecting the other functions of the plant. No appreciable effect is seen on the addition of an M/20 solution of NaCl. The presence of an M/10 solution on NaCl, however, reduces body-production from V to III in an otherwise favorable medium. Increasing this concentration to M/2 of the NaCl completely inhibits the production of the bodies.

AN ALBINO MUTATION

Brachysporium is a genus of the Dematiaceae, and is characterized by dark brown, sometimes almost black, mycelium and conidia. The form under consideration, *Brachysporium trifolii*, was isolated from infected plants of white clover in October, 1919. All cultures were started from single

spores at that time. These have been kept going in culture as a pure line since that time, with frequent transfers to new media, with as many as 75 parallel cultures at one time on various media. When grown on various sorts of agar media, for example, the dark brown or blackish color is characteristic of all of them. On cornmeal agar the color is purplish black. On oatmeal agar a very similar color is developed. In liquid media, of different kinds, whether synthetic or composed of extracts of natural products, the growth is always densely black, tending to dark gray in the aërial portions; this is also its habit on the natural host.

In October, 1921, a little more than two years after first growing it on artificial media, a series was planted on oatmeal-agar cups. In one oatmeal-agar cup, of a set of sixty-seven cultures started at that time, there appeared a variation from the normal dark color. From the point of inoculation in the center of the agar cup, a sector grew out which lacked the usual color character. There was, in this sector, a growth of floccose hyphae, as is the habit for the fungus, and this grew out over the agar and down the sides of the cup in a manner quite like that of the normal type except that it was completely lacking in the dark color. The sector was completely colorless. After the usual period of time there appeared in the culture the stalked bodies which develop on this medium, and which, in the normal form, are densely black. They also appeared in the usual manner in the colorless area, but like the mycelium were absolutely devoid of the dark color. They were white to light flesh-colored instead.

Sub-cultures were made from the contrasting area of this culture, by transferring some of the white stalked bodies to petri dishes containing sterile agar. A number of these bodies, before being planted on the new agar dishes, were washed through three changes of sterile distilled water. This was done to remove any chance conidia from the blackened area which might have been carried over to the colorless sector in the handling of the culture. A pure albino growth was obtained from these planted bodies. These cultures were made on November 17, 1921, and this albino strain has remained unchanged in character up to the present time (May 1, 1922). Pedigreed cultures have been made, and the plant has been carried through seventeen non-sexual generations in culture. The conidia produced by this albino strain correspond in size and shape with those of the normal strain. Repeated single-spore isolations have been made, and these spores germinate readily, in a manner characteristic for the normal dark-colored form of the plant. These single-spore cultures remain constant for the character of albinism, as do the cultures made by isolating bits of mycelium.

The general cultural reactions of this albino form correspond in every way with that of the normal black form. The requirements for conidium-production, for production of bodies, and for vegetative growth are in general the same as are those for the normal type.

This sudden and striking change occurring once in a series of several

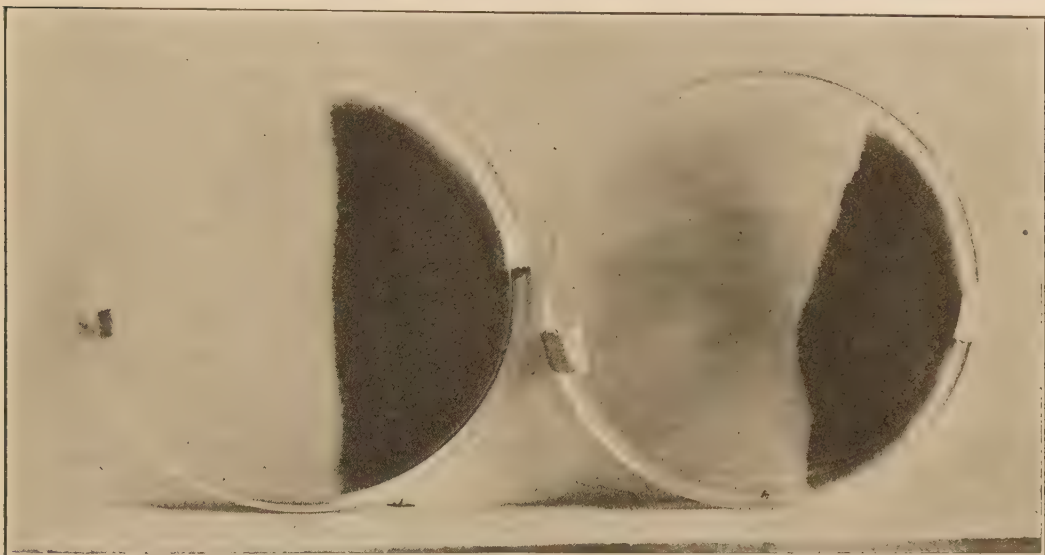
hundred cultures, and, showing so complete a transition in a single fundamental character of the organism, that of the dark color, will fall, it seems, under the category of a *bona fide* mutation.

A number of examples are cited in literature of mutations in fungi. These have in most cases had one general character in common, *viz.*, a dwarfing or reduction of development in the plant in some way. In some of these cases there is recorded simply a reduction in size or mass of growth of the fungus, while in other respects it retains the complete functioning of all its processes. Examples of such are mutants described by Schouten (1913) from *Rhizopus Oryzae*, and from *Phycomyces nitens*. Blakeslee (1920) describes what he calls "mutant A," from the hermaphroditic fungus *Mucor genevensis*. This mutant A differs from the parent form in having apparently lost its capacity to form zygospores. Blakeslee in the same article describes another type of mutant from the same species of *Mucor*. This second, "mutant X," is an example of a very decided dwarfing, so that all reproductive activity is lost, and only a reduced vegetation remains to represent the life history of the form. Schouten (1913) also describes one of this type: a mutation from *Dematium pullulans*, which not only lacks the capacity for reproduction, but also lacks the dark color of the normal type. These phenomena have all occurred in non-sexually propagated plants. They add, as Blakeslee points out, to the evidence that mutations are not restricted to processes involved in sexual reproduction.

Our case in hand, likewise, is an example of a mutation in a form which lacks any functional sexual process in its life history. This mutation is, however, somewhat different from those mentioned above. It is more clear-cut and striking, in that it is a change in one and only one factor, that of color. It does not involve a general reduction of growth or of some other life process. Usually such changes seem to have affected a group of factors. This appearance of an albino strain is a factor mutation. There simply seems to have appeared at one point in the formation of a new cell an irregularity, with the result that the new daughter cell did not carry the factor for color which is normal for this form. The new cell does, however, carry all the other factors for normal growth, asexual reproduction, etc., as is shown through a series of non-sexual generations studied in culture. This newly formed cell has not the factor for color, hence none of the subsequent growth from it can have the color, according to the indications of our knowledge and experience at the present time.

The contrasting appearance of this albino strain, which I have called strain A, with the original normal strain 1, from which A arose, is well shown by the photograph in text figure A. Although the mycelium of strain A does not show in the photograph in any way, the whole petri dish was covered by the growth of the two strains. These cultures were made by inoculating clear cornmeal-agar plates with the two strains, on opposite sides of the plate at the same time. Growth extends from the point of inoculation in a

normal manner in both strains until they meet. A sharp line is formed by this meeting, and this line remains fixed. The black area does not encroach on the colorless area by later growth. The growth of strain *A* seems slightly more extensive than that of strain *I*, when averaging a large number of comparative cultures. There is slightly more aërial mycelium, however, in strain *I* than in strain *A*.



TEXT FIG. A. Cultures showing the contrast when strain *I* and strain *A* are planted at opposite sides of a plate of oatmeal agar.

Studies were made to determine, if possible, whether strains varying in some other way might be found in this species. The fact that this strain *I* had been grown in culture for more than two years, under a great variety of conditions, without having formed any mature fruit bodies, suggested the possible existence of a sexually different strain. Single-spore isolations were made from the original infected clover, which was collected at Washington, D. C., July, 1919. The spores of this material germinated in a typical manner after two days, and thirty-five single-spore isolations were made. These were designated as strains *B-Z*, and 2-11, respectively. Each of these thirty-five strains, each of which had come from a single spore, was plated on oatmeal-agar plates opposite to strain *I* and allowed to grow against it. These platings were repeated three times and seventy-five other promiscuous platings were made, using any two strains which might be taken. The result of this was that in no case was there any reaction toward reproductive activity induced by the meeting of the two strains. This shows that in those strains used there is no sexual differentiation. Each produces the usual growth of conidia and stalked bodies all over the substratum.

One very noticeable difference has, however, been brought to light in this series of cultures. This difference consists in the great excess of aërial mycelium formed by cultures of strain 1 as compared with cultures of the thirty-five newly isolated strains. Strain 1 forms a heavy, felty mat of mycelium all over the surface of the petri dish, sometimes as much as 5 mm. in depth. This mat tends to hide the stalked bodies from view in many cases.



TEXT FIG. B. Culture showing the contrast when strain 1 and strain *R* are grown, starting at opposite sides of a petri dish.

The bodies are, moreover, fewer in number than in the newly isolated strains. There are comparatively few normal upright conidiophores on this excessive growth of mycelium, and conidium-production as a whole is less abundant than on the newly isolated strains. In the more recently grown strains, an abundance of upright conidiophores cover the surface of the agar. These arise from a thin layer of mycelium over the surface of the agar. The felt-like growth of mycelium is of a gray color in some cultures, and is much lighter in color than the mycelium next to the agar.

Text figure B is a photograph of a six-weeks-old culture of strain *R* on one side of the dish, and strain 1 on the other side.

When strain *R* was planted on both sides of the dish, there was a uni-

form growth over the whole surface of the dish and no contrast could be seen. There was a uniform growth characteristic of strain 1 when it was planted on both sides of the dish. Any two of the recently isolated strains, picked at random from the 35, showed, when plated opposite each other, a uniform growth over the agar, and no difference appeared in the growth of the strains of the same age in culture.

Strain 1 had been in culture for 28 months at the time that the contrast cultures were made. Each of the other strains had been in culture for two months. Of the thirty-five newly isolated strains, no cultural differences could be detected between any of them. They are exceptionally uniform when considered through the series of cultures used as a whole. However, the effect on strain 1 of long-continued culture on rich substrata has been to cause a general reduction of the asexual reproductive activity and a concomitant increase in vegetative growth.

FURTHER STUDIES ON PATHOGENICITY

In the earlier report on this fungus (Bonar, 1920), some results of inoculation experiments were recorded. Those experiments had been made during the winter months of 1919-1920. The fungus was kept growing in pure culture, and in the autumn of 1920 further inoculation studies were begun. At this time a number of different species of *Trifolium*² were included in the experiments. The species used were: *T. incarnatum*, *T. pratense*, *T. hybridum*, *T. dubium*, *T. procumbens*, and *T. arvense*. When these had attained a vigorous growth in pots, they were placed in an inoculation chamber. This inoculation chamber consisted of a large glass-sided aquarium chamber with a loose-fitting glass plate cover. The humidity of this chamber was maintained at a high degree by keeping a small amount of water on the floor of the chamber. The pots of clover were placed in this chamber with their bases raised above the water level. Check plants grew luxuriantly in this chamber for three months' time, although they wilted severely when transferred to the benches in the greenhouse. Pots of the above-named species in a vigorously growing condition were placed in this chamber in January, 1921, and sprayed with a heavy suspension of conidia from a pure culture of *Brachysporium trifolii*. This strain of the fungus had been isolated from white clover in October, 1919, and had been grown on rich media, with frequent transfers, until the time when these inoculations were made. At the end of two weeks a very slight infection was apparent on the Landino variety of *T. repens* and on *T. dubium*. Final results were recorded at the end of three weeks as follows: *Trifolium pratense*, no infection; *T. incarnatum*, 1-3 percent of leaves and petioles infected; *T. hybridum*, 5 percent; *T. repens* (Dutch White), 5 percent; *T. repens latus* (Landino var.), 15 percent; *T. dubium*, 5-10 percent; *T. pro-*

² The writer is indebted to Dr. A. J. Pieters, of the U. S. Department of Agriculture, for seed of a number of species.

cumbens, 5 percent; *T. arvense*, no infection. The percentage of infection was estimated by the number of leaves and petioles that had been killed. The cause of this killing was proven by the presence of the typical growth of *Brachysporium* spores on the infected parts.

It appeared from these experiments that the fungus was only slightly pathogenic for *Trifolium repens* (Dutch White), which was used to demonstrate its pathogenicity in the experiments reported in the former paper. The Landino or *latus* variety of *Trifolium repens*, commonly grown in some parts of Italy, showed a greater degree of susceptibility to the fungus than did the commonly grown Dutch White variety of this country. A repetition of the experiment, using these two varieties, showed this to be true to an even more marked degree than was shown by the first trial. A very decided decrease in the pathogenicity of the fungus is shown when these results are compared with those of the experiments reported in the previous paper and carried on in 1919.

New cultures were then made from single spores isolated in January, 1922, from the original clover from Takoma Park, Maryland. This material, collected in July, 1919, had lain in herbarium pockets in the desk in the laboratory from October, 1919, until January, 1922. Inoculation experiments similar to those described above were made in February, 1922, using as inoculum spores from this newly isolated material, which had been cultivated on artificial media for six weeks. The species used in these last inoculation experiments were: *T. incarnatum*, *T. pratense*, *T. hybridum*, *T. repens* (Dutch White), *T. repens latus*, *T. dubium*, and *T. spumosum*. At the end of one week numerous yellowed leaves were to be seen on some pots. They were more evident on *T. spumosum* and *T. repens latus* than on any of the other species. At the end of two weeks from the time of inoculation the leaves of many plants had fallen to the ground, and examination of these dying parts with the microscope showed them to have a plentiful growth of *Brachysporium* conidiophores and conidia over their surfaces. At the end of the third week, records were made on the various species as follows:

T. incarnatum: 80 percent of the leaves and petioles dead. They had fallen as a brown mass, and examination showed them to bear a great abundance of *Brachysporium* conidia.

T. pratense: 25 percent of the leaves infected, and either fallen or partly so. Often only one leaflet of the three was killed. Conidia were abundant on the dead parts.

T. hybridum: 50 percent of the leaves and upper portions of petioles infected. Infection in many cases did not extend to the base of the petiole. Conidia were abundant on the dead parts.

T. repens (Dutch White): 20 percent infection. Complete collapse of infected parts. Conidia were abundant on the dead parts.

T. repens latus: 75 percent infection. Infection was more rapid than in other varieties. Conidia were abundant on the dead parts.

T. dubium: 60 percent infection. Some stalks were completely killed; others showed scattered dead leaves.

T. spumosum: Infection 100 percent; 90 percent of the leaves and petioles were killed. The leaves sometimes fell over while still green and healthy in appearance because of the collapse of the supporting petiole near the base. Conidia abundant.

Control plants were in good condition, showing no signs of any infection.

Plants of *T. repens* (Dutch White) and *T. repens latus*, which were sprayed with a suspension of *Brachysporium* spores and allowed to remain uncovered on the bench in the greenhouse showed no infection. A pot of *Trifolium dubium*, when sprayed with a suspension of conidia from strain A (albino) of *Brachysporium trifolii*, and kept under a bell jar, failed to show any infection.

It is evident from these experiments that the newly isolated strain is more vigorously pathogenic than the one that has been in culture continuously for twenty-eight months. This fungus is also shown to be more pathogenic, under the conditions of the inoculation chamber, to other species of *Trifolium* than it is to the Dutch White clover commonly distributed through our northern states. The most susceptible species is found to be *Trifolium spumosum*, while *T. incarnatum* and *T. repens latus* show a high degree of susceptibility.

A very high degree of humidity and a temperature of at least 75 to 80° F. are necessary conditions for infection. The following experiment demonstrates the effect of humidity on infection. A pot of *T. repens latus* was set on a board, sprayed with a suspension of *Brachysporium* spores, immediately covered by a bell jar, and allowed to stand for two weeks. A second similar pot was likewise sprayed with a spore suspension. This second pot, with its base protected by a glazed porcelain saucer, was set on a broad plate of somewhat greater diameter than the bell jar used for a cover. About a quarter of an inch of water was kept in the large plate. At the end of two weeks, the pot which rested on the board showed no infection, and the soil in the pot was dry. The plants showed definite signs of their need for water. In the second pot a very noticeable infection had taken place, and the air within the bell-jar chamber was highly saturated with moisture. The earth in which the plants were growing, however, had not received any addition of water. This demonstration of the high degree of humidity necessary for infection, coupled with the facts concerning the relation of temperature to spore germination given earlier in this paper, correlates readily with the results of the inoculation experiments. Given a high degree of saturation of the atmosphere and a comparatively high temperature, infection is possible on a number of species of the genus *Trifolium* when a recently isolated strain of the fungus is used for the experiment.

It becomes evident, from these results, that this fungus is not likely to be of considerable importance from an economic point of view. The con-

ditions ordinarily met in the open field will not fall within the range of those which are necessary for the infection of clover plants by *Brachysporium trifolii*. This opinion is corroborated by Dr. A. J. Pieters of the U. S. Department of Agriculture. He, using a culture of the fungus furnished him by the writer, failed to secure positive results in attempted inoculations in fields of white clover in the spring of 1921.

GENERAL DISCUSSION

The studies reported in this paper give a record of the life processes of *Brachysporium trifolii*, and, in more or less detail, of the factors conditioning the development or lack of development of the plant and its reproductive organs. The fungus presents, for the most part, no unusual morphological features as regards its vegetative growth or its asexual reproduction. The production, however, of large numbers of the peculiar stalk-like bodies on some substrata is worthy of more special consideration, since it was the elucidation of what these bodies represented that concerned the writer during the greater part of the experimentation. The question of the significance of these bodies in the life history of the organism is clouded by their failing to perform any evident function.

These bodies resemble sclerotia in the early stages of their development. The inner homogeneous medulla-like tissue, and the outer rind of thick-walled cells of a young body, correspond to the structure of a sclerotium. These can not, however, be sclerotia, since they, by further development, exceed the category of structures found in a sclerotium. De Bary (1887, p. 42) speaks of a "motley assemblage" of such "fungous bodies" which may later change by differential development. The formation of special interior areas, which are morphologically distinct from the surrounding tissue, argues in favor of their being the immature stage of some reproductive body. The method of development, by replacing the original tissue with a special growth of differentiated cells, precludes the possibility of its being the early development of a pycnidium. A number of workers have given us information on the structure and development of pycnidia (Reddick, 1911; Kempton, 1919). No record of any process of differentiation such as occurs in these bodies has been found in the literature on pycnidium-development. The cells of this interior area arise by differential growth from what was formerly homogeneous tissue, to form a special area of differential cells bounded in part at least by a distinct wall.

This development can, however, be compared with some of the recorded developments in the perithecia of certain Pyrenomycetes. Blackman and Welsford (1912) say that the ascogenous hyphae of *Polystigma rubrum* arise by differentiation from vegetative mycelium. They maintain that this occurs after the disintegration of a sexual apparatus consisting of an ascogonium and antheridium, which, according to them, are non-functional. Nienburg (1914), working on the same plant, shows by his figures a fertili-

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zation process, but fails to show whether this has any relation to the formation of the ascogenous hyphae. Brooks (1910), in a study of *Gnomonia erythrostoma*, found, early in the development of the perithecium, what he considers to be an ascogonial apparatus. He did not, however, see any evidence that fertilization occurs. These cells disintegrate, and hence do not give rise to the ascogenous hyphae. They appear to arise late in the development of the perithecium after it is a well-formed homogeneous body of vegetative cells. Cayley (1921), in studies on the perithecia of *Nectria galligena*, makes a similar observation as to the origin of the ascogenous hyphae from apparently undifferentiated cells, rather than from any specialized sexual apparatus such as is known for *Ascobolus* or *Pyronema*. Reddick (1911) found that the pycnosclerotia of *Guignardia bidwellii*, which often remain sterile for a long time, may eventually develop into perithecia. These pycnosclerotia have certain central differentiated cells which show a much more pronounced staining reaction than the surrounding tissue. Noack (1905) definitely proved, by culture studies, that the bodies which had long been regarded as sclerotia in cultures of *Helminthosporium gramineum* were in fact immature perithecia of *Pleospora*, although Ravn (1901) had been able to secure only sterile growth after eighteen months in culture. From a consideration of these facts, I am led to the conclusion that the differential development in these stalked bodies in my cultures, represented in figures 9 and 10, Plate III, corresponds either to the early stages of the development of a perithecium, or to the development of an abortive body in part homologous with a perithecium. This disintegration continues until the protoplasm of these cells has completely disappeared (fig. 12). Although hundreds of these bodies have been carefully examined, no instance has been found of a more complete development of these perithecial primordia.

In search of another possible line of evidence on this subject, many young cultures, one to two weeks old, have been examined. It was thought that there might be some initial development on the agar which could be used to determine the origin of the development of the body as a whole. Brooks (1910) described the development of a coil-like structure as the initial of the perithecium of *Gnomonia erythrostoma*. My examinations, however, yielded only negative results.

Another line of argument which strongly supports the idea that these bodies are immature perithecia is afforded by a consideration of the physiological reactions of the plant as a whole. One of the principles most strongly brought out by Klebs (1899) is that the working limits of the general life conditions for a fungus are narrower for reproduction than for growth. He also goes on to show that the limits of conditions for the higher or sexual forms of reproduction are narrower than for asexual reproduction. He uses, as material for the support of this argument, data on *Sporodinia grandis*, *Saprolegnia mixta*, certain yeasts, and *Pleospora Sarcinulae*, as well as some others. This notion has been verified also for a large number of other

fungous forms. Other examples illustrating this same principle in perithecium-forming fungi are *Pleospora trichostoma* (Noack, 1905) and *Valsa leucostoma* (Leonian, 1921). A consideration of table 1 of the present paper shows that a large number of different substrata are favorable for at least a fair vegetative growth of this fungus. Table 2 shows that a considerably smaller number of these substrata are favorable for the production of conidia. Table 3 shows that a very much smaller number afford suitable food for the production of stalked bodies. The same relation holds for the other conditions of cultures as for the nature of the food stuffs mentioned above, e.g., temperature range, acidity or alkalinity, humidity, or the presence of some toxic substance. In this way the reactions of these stalked bodies are shown to correspond, in their general physiological behavior, to those known for the higher or sexual stage of reproduction of a considerable number of well studied forms. These facts, I think, support very strongly the argument for classing these stalked bodies as perithecia which for some reason do not attain a full development.

A number of cases are known in which complete development of Ascomycetes has been secured by employing some one special factor to fulfill what are otherwise incomplete conditions for development. Noack (1905) found that the sclerotia in cultures of *Helminthosporium gramineum* are stimulated to development into perithecia by exposing the cultures to a low temperature for a short time. Cultures of *Brachysporium trifolii* showing well developed, vigorously growing bodies have been placed in refrigerators at 0° and 10° C. and left for varying lengths of time up to one year. But neither after this treatment nor when they are again put under a higher temperature, is any more complete development found. Saito (1918) found that the addition of 4 to 10 percent NaCl to cultures of *Zygosaccharomyces major* stimulated the production of asci. When, however, NaCl was added to my cultures, there resulted an inhibition of the formation of the bodies, as is recorded earlier in this paper.

Cayley (1921) finds that the presence in the culture medium of a small percentage of glycerin is necessary for the formation of the ascus stage of *Nectria gallegina*. The addition of 0.5 to 1 percent glycerin to the cultures in my study absolutely inhibited all production of the bodies. Leonian (1921) found that the addition of sugars to a rich oatmeal agar induced the formation of perithecia in *Valsa leucostoma*. In *Brachysporium*, however, the addition of levulose, although it induces a more vigorous and prolonged general development of the plant, does not produce a greater degree of differentiation in the stalked bodies. Sucrose produces a similar effect, although the stimulation is slightly less pronounced than when levulose is used.

The existence, within a single fungous species, of complementary strains, the presence of both of which is necessary for sexual development, has been demonstrated for a wide variety of forms. Blakeslee (1904) designated

these as "plus" and "minus" strains when he first discovered them in the Mucorales. Edgerton (1914) and Dodge (1920) report the existence of complementary strains in the Ascomycetes. Many cultures were therefore made, as described earlier in this paper, to test the possibility of securing a reproductive reaction between two different strains. None such was secured, nor did any indication appear of any tendency toward such a reaction. Blakeslee (1906) says, when speaking of algae and fungi:

The absence of sexual reproduction may be due: (1) to constitutional sterility; (2) to conditions of growth unfavorable to the production of sexual organs; or (3) to the fact that the form is heterothallic and that the thalli of both sexes have not been brought together.

The first category hardly covers the material under consideration, since a very plentiful development of the bodies to a fairly advanced stage is obtained. The large number of variations of conditions employed in this culture work tends to throw the weight of evidence away from the second possibility. All of my experiments to show that the third supposition might apply to the fungus under study have, likewise, given negative results.

After considering all the facts concerning the nature of these bodies which develop in cultures, we are led to the conclusion that they represent a degenerate stage of what was once a perithecial form of reproduction.

A very interesting example of the attenuation of the virulence of a fungus through long-continued culture on artificial media is afforded by this organism. Krakover (1917) says:

It is commonly asserted that parasitic fungi lose their virulence when kept in culture for a long time, but exact experimental evidence is not at hand.

He gave an example of attenuation in *Macrosporium sarcinaeforme* from red clover. The attenuation of the virulence of *Brachysporium trifolii* after continued culture, as discussed earlier in this paper, is coincident with an increase in vegetative growth at the expense of reproductive activity. There seems to have occurred, in this long-cultivated strain 1, a certain reduction in the scale of its life activities. There has been a loss of virulence and of some of its reproductive activity, both of which are more specialized characteristics than mere vegetative growth, which has been increased. This is shown to be true when these later cultures of strain 1 are compared with its original virulence and with its original habit of growth in culture. It is also shown to be true when compared with the thirty-five recently isolated strains from the original collection of material, even after the latter has been dried for a long time. This variation is not to be placed in the same category with a mutation nor confused with that idea. An example of a mutational change is afforded by strain A. This mutation represents a clear and distinct change of a definite nature, comprising all the morphological structures of the fungus, which occurs suddenly and remains distinct without any apparent other change or changed relations to any of the regular life processes of the organism.

SUMMARY

1. *Brachysporium trifolii* Kauff. has been studied with regard to its physiological reactions and its complete life history both in culture and on its native host.

2. Very numerous slender black bodies are produced in certain cultures. These bodies are at first composed of homogeneous pseudoparenchymatous tissue. Later growth shows a differentiation of definite interior areas in this homogeneous tissue. These interior areas resemble the early development within a young perithecium. At a certain stage, a disintegration of the protoplasm sets in within the cells of this inner area, and the result is a sterile tissue which undergoes no further development.

3. From among a large number of food materials used, heavy oatmeal agar is the most favorable for the production of the stalked bodies.

4. The development of the mycelium, conidia, or bodies is to a very large degree conditioned by the environment under which the fungus is grown. The organism is found to have a wider range of conditions suitable for vegetative growth than for asexual reproduction. The conditions for asexual reproduction are, likewise, much more general than those for the production of the stalked bodies. This relation of the different reactions corresponds with the reactions of fungi in general.

5. Considered from the standpoint of morphology, method of development, and general physiological reactions, the stalked bodies seem to be immature perithecia, or, in part at least, homologous with perithecia.

6. A distinct albino mutation has been found once in a long-cultivated pure line of the normal dark-colored form.

7. Long-continued growth of the fungus in culture causes an attenuation of its virulence.

8. Different species of *Trifolium* show varying degrees of susceptibility to attack by *Brachysporium trifolii*, under the conditions of the inoculation chamber.

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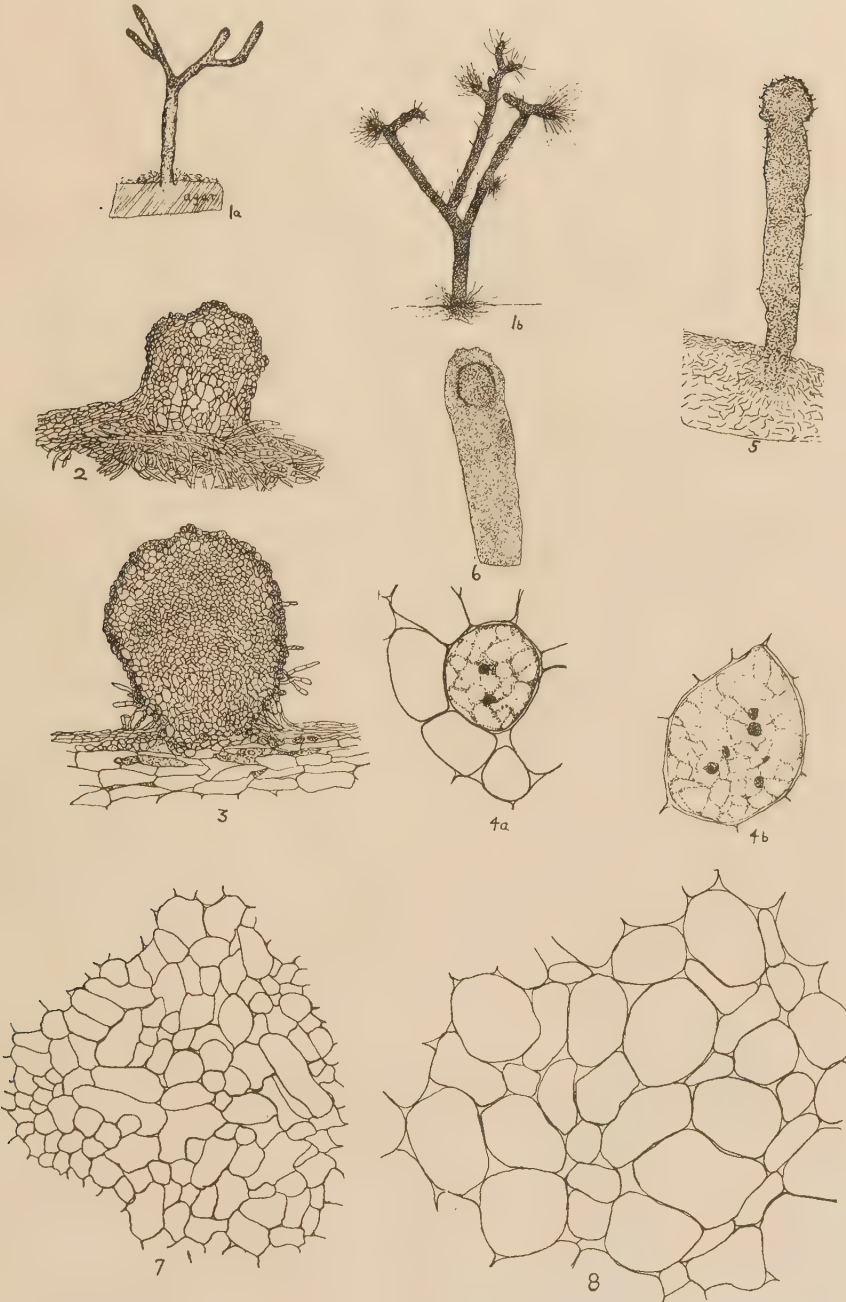
DESCRIPTION OF PLATES

PLATE II

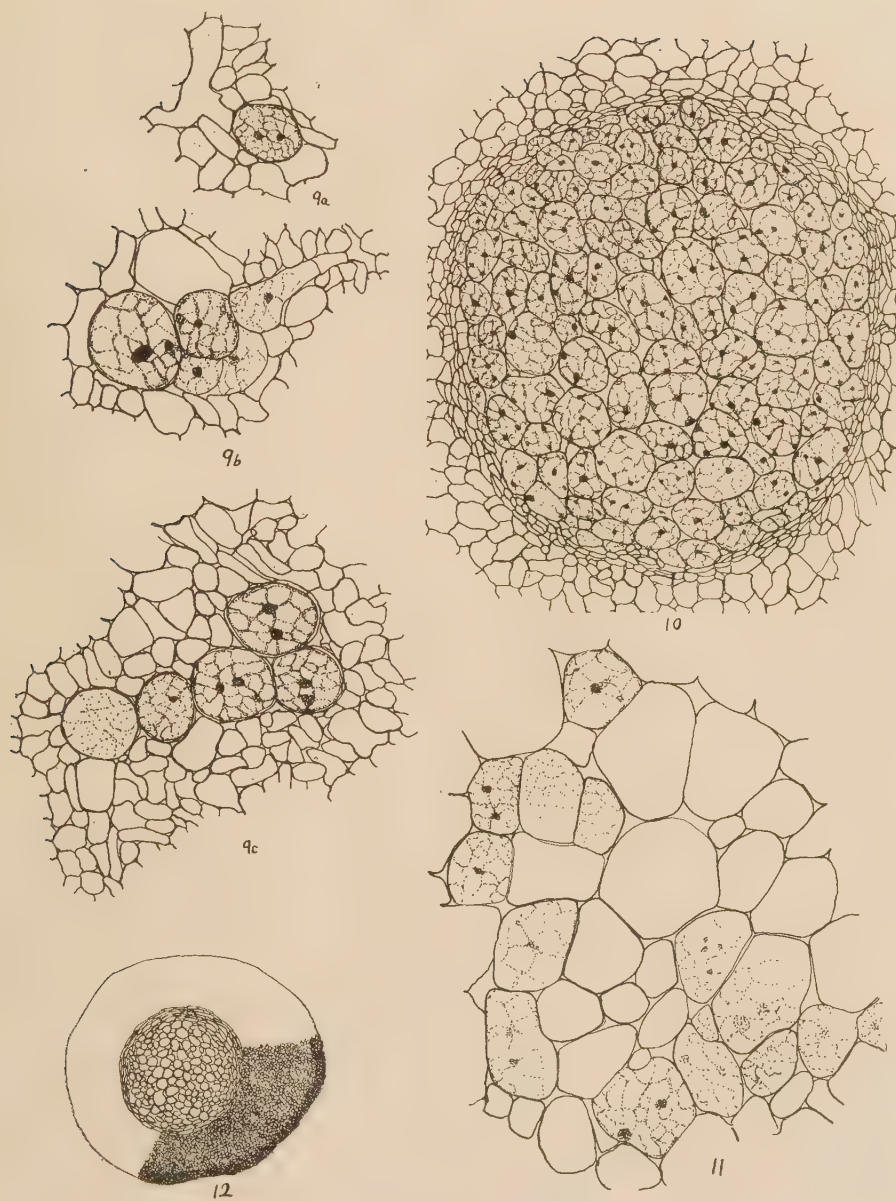
- FIGS. 1 *a, b*. *a*, one of the stalked bodies 45 days old; *b*, one 60 days old. $\times 5$.
- FIG. 2. Longitudinal section of a young body, showing its relation to the agar from which it grew. $\times 125$.
- FIG. 3. Longitudinal section of a young body, showing its origin on a stem of *Melilotus* on which it was grown. $\times 125$.
- FIGS. 4 *a, b*. Cells from the young bodies shown in figures 2 and 3. $\times 1425$.
- FIG. 5. Longitudinal section through the length of one of the bodies 25 days old. $\times 32$.
- FIG. 6. Longitudinal section through the tip of a body 60 days old, showing the differentiated area within. $\times 32$.
- FIG. 7. Cells from the undifferentiated portion of a 60-day-old body. $\times 1260$.
- FIG. 8. Cells from the inner differentiated area of the same body shown in figure 7. $\times 1260$.

PLATE III

- FIGS. 9 *a, b, c*. Stages in the differentiation and development of the inner areas from the ordinary body cells. $\times 1260$.
- FIG. 10. Further development of a differentiated area and the formation of the limiting wall. $\times 1260$.
- FIG. 11. Disintegration of the protoplasm of the cells in the inner area, after the greatest recorded development has been attained. $\times 1425$.
- FIG. 12. Cross section through one of the bodies, showing the different areas after there has been a complete loss of all the protoplasm from all the cells. $\times 125$.



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